

## Research Article

# Ulvan, a Sulfated Polysaccharide from Green Algae, Activates Plant Immunity through the Jasmonic Acid Signaling Pathway

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The industrial use of elicitors as alternative tools for disease control needs the identification of abundant sources of them. We report on an elicitor obtained from the green algae *Ulva* spp. A fraction containing most exclusively the sulfated polysaccharide known as ulvan-induced expression of a GUS gene placed under the control of a lipoxygenase gene promoter. Gene expression profiling was performed upon ulvan treatments on *Medicago truncatula* and compared to phytohormone effects. Ulvan induced a gene expression signature similar to that observed upon methyl jasmonate treatment (MeJA). Involvement of jasmonic acid (JA) in ulvan response was confirmed by detecting induction of protease inhibitory activity and by hormonal profiling of JA, salicylic acid (SA) and abscisic acid (ABA). Ulvan activity on the hormonal pathway was further consolidated by using *Arabidopsis* hormonal mutants. Altogether, our results demonstrate that green algae are a potential reservoir of ulvan elicitor which acts through the JA pathway.

## 1. Introduction

Plants have sophisticated mechanisms to perceive and defend against microbial pathogens. It is now widely accepted that early recognition of pathogens mainly relies on the detection of microbial (or pathogen) associated molecular patterns (MAMPs or PAMPs), formerly called elicitors. MAMPs are characteristic structures shared by large groups of microbes [1]. They are generally present in molecules that play a critical role in the lifestyle of the corresponding organism. A large diversity of molecules harboring MAMPs has been identified including proteins, polysaccharides, and lipids, and a few receptors able to specifically recognize MAMPs were also identified [2]. MAMP perception induces early events such as membrane depolarization, changes in intracellular calcium concentration [3], production of

active oxygen species, phosphorylation cascades by mitogen-activated protein kinase [4], and activation of signaling pathways depending on the phytohormones, jasmonic acid (JA), ethylene, and salicylic acid (SA). The importance of these molecules as primary signals in the regulation of the plant immune response is well established. More recently, auxins, gibberellins, cytokinins, and abscisic acid (ABA), hormones implicated on plant development and response to abiotic stresses, emerged as players during biotic stress as well, [5].

A major class of inducers of plant immunity consists of oligosaccharides such as  $\beta$ -1,3-1,6 glucans, and chitin-derived oligomers, which can be released from oomycetal and fungal cell walls during the interaction with their host plant [6]. These compounds are not restricted to microorganisms since they are also produced by marine

organisms, algae, and crustaceans, thereby providing an inexpensive source of biologically active molecules which can be potentially used as plant protectants. The  $\beta$ -1,6-1,3 glucan called laminarin, a storage polysaccharide from the brown alga *Laminaria digitata*, stimulates defense responses in a variety of plant species providing protection against pathogen attack [7, 8]. Besides these well-known oligosaccharide patterns, other algal polysaccharides were further shown to induce plant defense responses. Most of them carry sulfate groups, notably fucan and carrageenans, two sulfated polysaccharides from brown and red algae, respectively [9, 10]. They behave as typical MAMPs inducing rapid changes in intracellular calcium concentration [3, 11], an oxidative burst [8, 9, 12] and downstream defense responses such as induction of enzymes involved in the phenylpropanoid pathway and pathogenesis related proteins [9, 10, 12, 13]. The structure and composition of algal oligosaccharides were shown to be crucial for their activities on signaling pathways regulating plant defense. For example, sulfated oligosaccharides obtained from fucan and carrageenans, but not laminarin, were shown to induce the salicylic acid signaling pathway [9, 10]. The crucial role of sulfate groups was further demonstrated by chemical sulfation of laminarin [8, 9] leading to a compound that became an inducer of the SA signaling pathway in tobacco and of associated resistance to the tobacco mosaic virus [13].

In a previous work, we reported on the elicitor activity of a green algal crude extract on the model legume *Medicago truncatula* [14]. Green algae belonging to the *Ulva* genus are common seaweeds that are abundantly found worldwide generally proliferating in eutrophicated coastal waters [15]. The algal extract consisted of water soluble components, whose biochemical analyses revealed the presence of typical polysaccharides found in green algae, known as ulvan, suggesting that this molecule can act as a plant defense elicitor. The main constituents of ulvan are sulfated rhamnose residues linked to uronic acids, resulting in a repeated disaccharide unit  $\beta$ -D-glucuronosyl-(1,4)- $\alpha$ -L-rhamnose 3-sulfate, called aldobiouronic acid [16]. The *Ulva* extract was shown to induce a large set of defense genes upon treatment of *M. truncatula* leaves and to protect the plant against infection by the pathogenic fungus *Colletotrichum trifolii* [14].

In the present work, we have characterized the elicitors present in the green algal extract of *Ulva* spp. and performed in depth analysis of their activity through cellular and molecular approaches.

## 2. Materials and Methods

**2.1. Plant and Algal Material.** *Nicotiana tabacum* L. *P1lox::GUS* plants (obtained by B. Verdaguer, UMR 5546, Toulouse, France) were grown on soil in a growth chamber (16 hours light at 25°C, 8 hours dark at 22°C). Seeds of *Medicago truncatula* line F83005.5 (provided by J. M. Prospero, ENSAM, INRA Montpellier, France) were surface-sterilized for 5 minutes in sulphuric acid, rinsed several times with sterile water, and germinated on Petri dishes

for 2 d at 20°C in the dark after 1 night at 4°C. Seedlings were transferred to soil and cultivated in a growth chamber (16 hours light at 25°C, 8 hours dark at 22°C). *Arabidopsis thaliana* seeds of the ecotype Columbia (Col-0), *jar1-1*, *NahG*, and *aba3.1* mutants and the transgenic line *PRI::GUS* [17] were deposited on damp soil at 4°C during two days and transferred in a growth chamber (9 hours light at 22°C, 15 hours dark at 20°C) for 4 weeks. Green algae *Ulva* spp., a mixture of several *Ulva* species (mostly *U. armoricana*), were harvested on the north Brittany coast (France) at Archipel de Brehat by Biotechmarine in August 2004, washed with fresh water, and immediately frozen.

**2.2. Preparation of *Ulva* Extracts.** 100 g of water-washed algae were grinded and were autoclaved for 2 hours at 90°C (1.97 atm) in 1 l distilled water and the resulting extract was filtered through a nylon mesh (80  $\mu$ m porosity) on a sintered glass funnel (G2 porosity). The filtrate was lyophilized and designated as “crude extract”. In this extract, the compounds of high molecular mass were precipitated with 2.5 volumes of ethanol for 48 hours at -20°C. The supernatant and pellet were separated by filtration, lyophilized, and subsequently called “ethanol-soluble” and “ethanol-insoluble” fractions, respectively. Size exclusion chromatography of the ethanol-insoluble fraction was achieved on Sepharose CL 6B column (25  $\times$  26 cm) using 100 mM ammonium formate as eluent. The column was calibrated with standard dextrans (10–1500 kDa). The 600–400 kD fractions were pooled and lyophilized. In a separate experience, 20 mg of the ethanol-insoluble fraction were dissolved in 5 ml of 100 mM ammonium formate, treated by ultrasounds (20 kHz, 5  $\times$  3 minutes) and then submitted to size exclusion chromatography. The 60–40 kD fractions were selected using the same conditions as above.

**2.3. Biochemical Analyses of *Ulva* Spp. Extracts.** Total uronic acid content was determined according to Blumenkrantz and Asboe-Hansen [18] using glucuronic acid as a standard. Neutral sugars were released by hydrolysis in 2 M TFA (120°C, 1 hour). TFA was eliminated by evaporation. Sugars were analyzed by HPAEC-PA on anion exchange column (Dionex, CarboPac MA1) with suitable standards. Sulphur measurements were done using inductively coupled plasma atomic emission spectroscopy (ICP-AES).

**2.4. Infiltration Assays and Plant Treatments.** Fully expanded leaves of 5-week-old *N. tabacum* were infiltrated with water and fractions from the green algae (1 mg·ml<sup>-1</sup>). Infiltrated tissues from at least 5 plants per treatment were collected 3 days after treatment. In the case of *PRI::GUS* transgenic *Arabidopsis* plants, fully expanded leaves of 4-week-old plants were infiltrated with water, the ethanol-insoluble fraction (1 mg·ml<sup>-1</sup>) and P2 (500  $\mu$ g·ml<sup>-1</sup>), a *Phytophthora parasitica* elicitor cell wall extract [19]. Four-week-old *M. truncatula* and *A. thaliana* plants were treated with sterile water, Actilandes (Samabiol, France) at 0.01%, MeJA (500  $\mu$ M) dissolved in Actilandes (0.01%), ethanol-insoluble ulvan extract (1 mg·ml<sup>-1</sup>) dissolved in Actilandes

(0.01%), and the salicylic acid analog acibenzolar-S-methyl (BTH,  $40 \mu\text{g}\cdot\text{m}^{-1}$ ). Solutions were sprayed onto plant leaves (2 ml per plant). For *M. truncatula*, a time course experiment was carried out at 1, 2, and 6 days and leaves from 9 plants per time point were collected. In the case of *A. thaliana*, leaves from 3 plants were sampled at 5 days.

**2.5. GUS Activity Assays.** Proteins were extracted from infiltrated tissues of tobacco or *Arabidopsis* leaves. Glucuronidase activity was measured on the protein extracts using a fluorometric assay [20]. Briefly, 100 mg of grinded tissues were lysed in 100  $\mu\text{l}$  of GUS buffer (100 mM Phosphate buffer, pH 7.0, 10 mM EDTA) with 0.1% TritonX-100 and 10 mM  $\beta$ -mercaptoethanol. The fluorogenic reactions were carried out in 1 mM MUG (4-methylumbelliferyl glucuronide, Sigma) in GUS buffer with 25  $\mu\text{l}$  of protein extracts in a reaction volume of 200  $\mu\text{l}$ . Fluorescence was measured every 5 minutes during 90 minutes, on an FL600 Microplate Fluorescence Reader (Bio-Tek Instruments) at 37°C with 360 nm excitation and 460 nm emission. The fluorimeter was calibrated with freshly prepared MU (4-methylumbelliferone sodium salt, Sigma) standards in the same GUS buffer. Protein concentration was determined by the method of Bradford on 96 well plates. 200  $\mu\text{l}$  of Bradford reagent (Bio-Rad Laboratories) were added to 10  $\mu\text{l}$  of samples. After incubation (15 minutes, room temperature), absorbance was measured at 565 nm on an ASYS Expert 96 (Isogen) spectrometer. Standard curve was done with 1–20  $\mu\text{g}$  of BSA (Sigma). Glucuronidase activity was calculated from the linear part of the reaction (between 10 and 60 minutes) and expressed as nkatal  $\text{mg}^{-1}$  of protein.

**2.6. RNA Extraction and Microarray Hybridization.** Total RNA from grinded leaf tissues was extracted using TRIzol reagent (Invitrogen) and purified using Microcon-30 columns (Millipore). 5  $\mu\text{g}$  of total RNA were used to synthesize Cy3- and Cy5-labeled cDNA targets using a mixture of 3  $\mu\text{g}$  of double anchored oligo (dT) 15 VN primers and 3  $\mu\text{g}$  of random primers with CyScribe First Strand cDNA labeling Kit (Amersham Biosciences). Labeled cDNA was purified using the ChipShot Membrane Clean Up System (Promega), and labeling efficiency was checked by measuring absorbance at 260, 550, and 650 nm with a Nanodrop (Thermo Scientific). Only labeled cDNA having a frequency of incorporation of at least 20 labeled nucleotides per thousand was used for hybridization. Microarray analyses were performed using Mt16kOLI1Plus chips represented all 16086 tentative consensus sequences of the TIGR *M. truncatula* Gene Index 5 plus a set of 384 oligonucleotides representing transcription factors and regulators. Automatic hybridization of a mix containing the same quantity of Cy3- and Cy5-labeled cDNA was performed in a Ventana Medical Systems room (Discovery) following the standard procedure. After 16 hours of hybridization at 42°C, microarrays slides were washed twice in RiboWash reagent (1 minute, 20°C) and once in  $0.1\times$  SSC (1 minutes, 20°C). Slides were dried by centrifugation (185 g, 5 minutes, 20°C) and scanned using the Genepix 4000 scanner (Axon Instruments). Image

processing was performed using the Mapix (Innopsys, France) software. Subsequently, the mean intensities of signal pixels and the mean intensities of background pixels were calculated for each spot in both channels. Spots were flagged “empty” in the case of  $R < 0.7$  for both channels.  $R$  is defined as the difference between the mean intensity of signal pixels and the mean intensity of local background pixels divided by the standard deviation of local background pixels. A saturated loop design was done for the two time points. Cy3 and Cy5 data were treated separately as one channel array. Normalization was done by local linear regression analysis (LOWESS) using the limma library of  $R$  software. All data were deposited in MIAME compliant format in the ArrayExpress database under the accession number E-MEXP-1995.

**2.7. Hormonal Profiling.** SA, JA and ABA and their internal standards  $^2\text{H}_4$ -SA,  $^2\text{H}_5$ -JA were extracted and measured in *M. truncatula* leaf tissues according to a protocol adapted from Forcat et al. [21]. Hormones were extracted from 100 mg frozen material, to which 40 ng of internal standards were added at the beginning of extraction. Briefly, two successive extractions with 400  $\mu\text{l}$  of 10% methanol containing 1% acetic acid were done on ice as described precisely. 5  $\mu\text{l}$  of the supernatant were then analyzed by UPLC-ESI-MS/MS using an Acquity UPLC coupled to a Micromass Q-ToF Premier (Waters, Massachusetts, USA) orthogonal acceleration time-of-flight (oa-TOF) mass spectrometer. Chromatographic separations were performed on a 100 mm  $\times$  2.1 mm  $\times$  1.7  $\mu\text{M}$  Acquity UPLC BEH C18 analytical column (Waters, Massachusetts, USA) maintained at 35°C. Temperature of the sample organizer was set at 4°C. The binary solvent system included A = water + 0.1% HCOOH and B = acetonitrile LC/MS grade. Gradient profile consisted of 0-1 minute: 5% B; 1-8 minutes: 5-68% B; 8-9 minutes: 95% B; 9-10 minutes: 95% B; 10-11 minutes 5% B, 11-12 minutes 5% B. Flow rate was 300  $\mu\text{l}/\text{minutes}$  and column eluent was directed to the mass spectrometer.

SA, JA, and ABA and their internal standards  $^2\text{H}_4$ -SA,  $^2\text{H}_5$ -JA were analysed as [M-H]<sup>-</sup>ions using electrospray ionization tandem mass spectrometry (ESI-MS/MS) in negative mode. The capillary voltage and cone voltage of mass spectrometer were set to 2.8 kV and 30 V, respectively. The source temperature was set to 120°C and the desolvation temperature to 400°C. Nitrogen was used as both cone gas (30 l/hour) and desolvation gas (800 l/hour). Argon was used as collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution. To ensure accuracy and reproducibility, analyses were acquired using the leucine-enkephalin in real-time as the lock mass at a concentration of 500 pg/ $\mu\text{l}$  and flow rate of 2  $\mu\text{l}/\text{minutes}$ . Data were acquired in centroid mode by MassLynx software. The data acquisition rate was set to 0.25 s, with a 0.02-second interscan and the lockspray frequency was set to 10 s and data averaging over 3 scans. Quantification was based on pseudo-MRM (Multiple Reaction Monitoring) after collision-induced dissociation. Collision energy was determined for each compound after infusion experiments



with individual phytohormone at 2 ng/ml, 15 eV for SA and  $^2\text{H}_4$ -SA, 12 eV for ABA, and 17 eV for JA, and  $^2\text{H}_5$ -JA. The MRM transitions for quantification were: 137.0 > 93.0 for SA, 141.0 > 97.0 for  $^2\text{H}_4$ -SA, 263.1 > 153.1 for ABA, 209.1 > 59.0 for JA, and 214.1 > 62.0 for  $^2\text{H}_5$ -JA. Quantitative results were calculated using QuanLynx software (Waters). To generate standard curves, 40 ng of internal standard were added to standards of SA, JA, and ABA (0.5 ng to 250 ng/ml). Analyte standard curves were calculated using QuanLynx and the area of the daughter ion of the sample to the daughter ion of the internal standard was determined (SA/ $^2\text{H}_4$ -SA; JA/ $^2\text{H}_5$ -JA and ABA/ $^2\text{H}_4$ -SA). Concentration of the sample analyte was then determined in relation to the internal standard in three independent samples for each treatment or time.

**2.8. Protease Inhibitor Activity.** Extraction of protease inhibitor was carried out according to Kunitz [22]. 1.5 g of *M. truncatula* leaf tissues harvested 6 days after treatment were ground in liquid nitrogen. The fine powder was suspended in 4.5 ml of 0.5 M Tris-HCl, pH 8.5, containing 0.5% (w/v) polyethylene glycol and 3% (w/v) polyvinylpyrrolidone and allowed to thaw under occasional shaking for about 1 hour in a cold room. The liquid was cleared by centrifugation (1 hour, 13000 g at 4°C). The supernatant was subsequently heated at 65°C for 10 minutes and the precipitate was removed by centrifugation (5 minutes, 10000 g). The extract obtained by this procedure was used for determination of protease inhibitor activity. 250  $\mu\text{l}$  of extracts were brought to a volume of 450  $\mu\text{l}$  with phosphate buffer, pH 6.8 in test tubes containing small magnetic bars and placed in a waterbath at 30°C where they were constantly stirred. 50  $\mu\text{l}$  of a trypsin solution (TPCK Cooper Biomedicals; 1 g·ml<sup>-1</sup> in 1 mM HCl) were added to each test tube except in the controls. After 3 minutes at 30°C, 500  $\mu\text{l}$  of 1% (w:v) casein “Hammarsten” (Merck, Darmstadt, Germany) were added and incubated at 37°C for 2 hours. After precipitation in 3% (w:v) TCA for 30 minutes in ice and centrifugation, absorbance at 340 nm of supernatant was measured. Proteins were detected by the method of Lowry et al. [23]. The protease inhibitor activity was expressed as trypsin ( $\mu\text{g}$ ) inhibited by plant proteins (mg).

**2.9. RT-Q-PCR.** Total RNA was extracted from 100 mg shock-frozen leaf tissues using the SV Total RNA Isolation System (Promega) and dissolved in 30  $\mu\text{l}$  of RNase free water. RNA quality was checked on agarose gel and possible DNA contamination was controlled by PCR. 2  $\mu\text{g}$  of total RNA were subjected to reverse transcription with SuperScript<sup>TM</sup> III (Invitrogen) with Oligo dT primers. Real-time RT-Q-PCR was performed on an ABI Prims SDS 7900HT (Applied Biosystems). Each 10  $\mu\text{l}$  PCR reaction contained 2  $\mu\text{l}$  a 1:50 dilution of the synthesized cDNA, 5X qPCR Master Mix Plus for SyberGreen I (Eurogentec), and primers (0.15  $\mu\text{M}$  each). The PCR program consisted in 5 minutes incubation at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 60°C. Primers efficiency was evaluated by performing real-time PCR on serial dilution of a mix of all cDNAs. Data normalization was

performed using the constitutive expressed gene encoding  $\beta$ -TUBULIN. The biological experiment was repeated 3 times. Gene-specific primers were designed with a calculated melting temperature of 60°C  $\pm$  0.5°C and a unique amplification not longer than 300 bp. The following primer sequences were used:  $\beta$ -TUBULIN, *At5g44340*, forward, 5'-GAGGGAGCCATTGACAACATCTT-3', reverse, 5'-GCGAACAGTTCACAGCTATGTTCA-3', *PR1*, *At2g14610*, forward, 5'-GGAGCTACGCAGAACAATAAGA-3', reverse, 5'-CCCACGAGGATCATAGTTGCAACTGA-3', *PDF1.2*, *At5g44420*, forward, 5'-TCATGGCTAAGTTTGCTTCC-3', reverse, 5'-AATACACACGATTTAGCACC-3'.

**2.10. Statistical Analyses.** We performed Student's *t*-test to determine probability that the two means, respectively associated to the control and tested populations, were equal. The statistical analyses were done using the R software (<http://www.r-project.org/>), where the *t*-test function was available from the stats library. We considered the two means significantly different when the *P*-value associated was weaker than .05.

### 3. Results

**3.1. Characterization of Ulvan as an Active Fraction from Green Algae.** Green algae *Ulva* spp., mostly *Ulva armoricana*, was treated with hot water to yield a crude extract containing mainly polysaccharides, which accounted for 60% of dried material. Ethanol precipitation allowed the separation of high, insoluble from low, soluble, molecular mass compounds. Size exclusion chromatography showed that the ethanol-insoluble compounds were mainly composed of two macromolecular populations (Figure 1(a)): a high molecular mass polysaccharide fraction (MW 600–400 kDa) and a low molecular population which was less defined. The 600–400 kDa fraction was sonicated in order to reduce the mean molecular mass of the polysaccharides and the resulting compounds were further fractionated on a Sepharose column. A major fraction containing polysaccharide fragments with a mean molecular mass of 50 kDa (60–40 kDa fraction) was selected for further characterization.

Uronic acid, neutral sugar and sulphur contents were determined in the crude extract and in all subsequent fractions (Table 1). The ethanol-insoluble fraction which represented 44% of the total material recovered from the crude extract contained uronic acids, rhamnose and sulfate as major components, consistent with the main constituents of ulvan, a known sulfated polysaccharide present in *Ulva* spp. [16]. Low amounts of xylose, galactose, and glucose residues were also found. In contrast, the ethanol-soluble fraction, which accounted for 56% of the crude extract, contained low amounts of sulphur and neutral sugars, while mannitol appeared to be the major component of the fraction (data not shown). The uronic acid, rhamnose, and xylose contents of the 600–400 kDa and 60–40 kDa fractions were higher than those of the ethanol-insoluble fraction; however relative amount of each molecules was conserved between the three fractions.

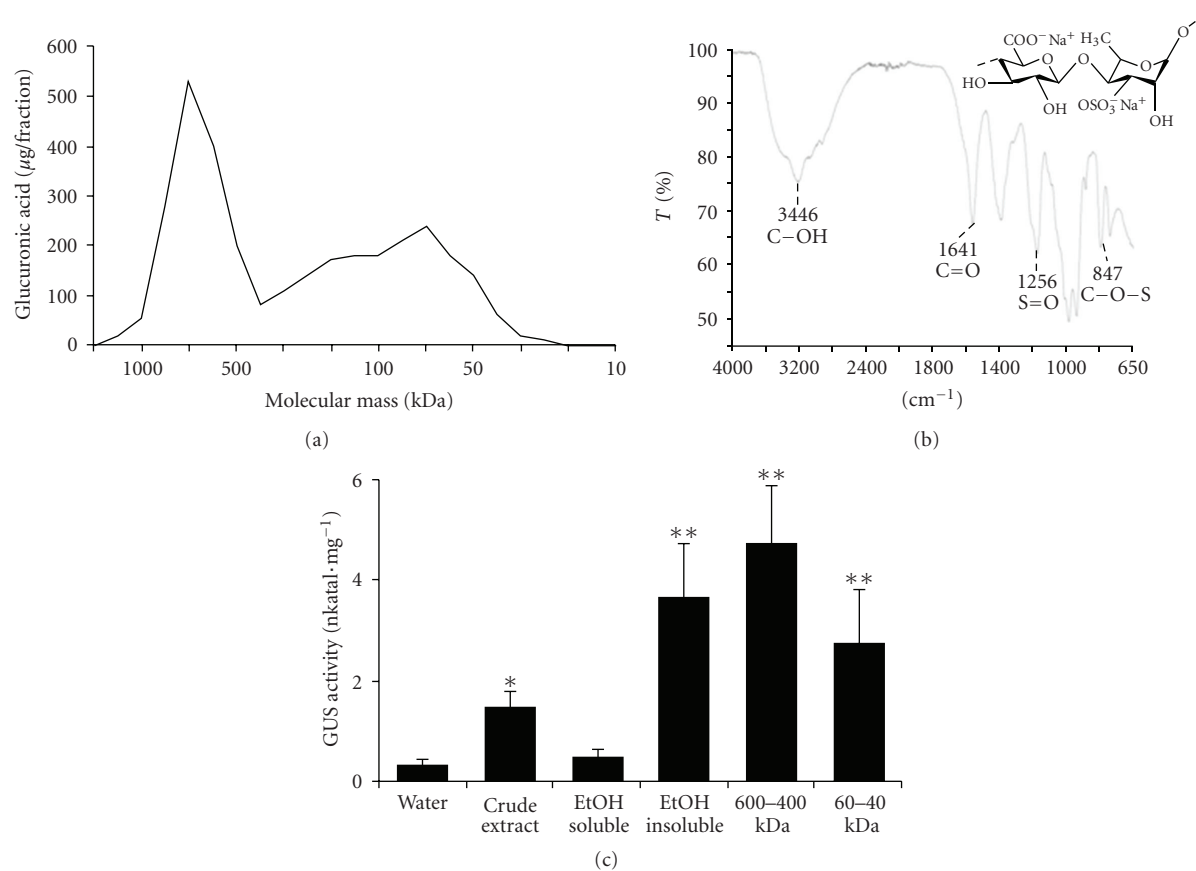


FIGURE 1: Biochemical analysis and biological activity of fractions from green algae *Ulva* spp. (a) Size exclusion chromatography of the high molecular mass compounds present in the ethanol-insoluble fraction recovered from the crude extract. Fractionation was followed by glucuronic measurement. (b) FTIR spectra of the 600–400 kDa fraction. The peaks at 847, 1052, 1256, 1641, and 3446  $\text{cm}^{-1}$  are respectively generated by the bending vibration of C–O–S in axial position, the stretching vibration of C–O, S–O, C=O, and C–OH, all linkages being present in the constitutive dimer  $\beta$ -D-glucuronosyl-(1,4)- $\alpha$ -L-rhamnose-3-sulfate. (c) Biological activity of the crude extract and fractions. GUS activity was measured on *P1Lox::GUS* transgenic tobacco 3 days after infiltration in leaves of the various samples ( $1 \text{ mg}\cdot\text{ml}^{-1}$ ) as indicated. Means, for each treatment compared to the control mean using a Student's *t*-test. Treatment associated to a *P*-value  $\leq .05$  or  $\leq .01$  was marked, respectively, by \* or \*\*.

TABLE 1: Chemical composition of an *Ulva* spp. crude extract and fractions obtained by ethanol precipitation and size-exclusion chromatography ( $\text{mg}\cdot\text{g}^{-1}$  of dry matter).

Fraction	Uronic acid	Neutral sugars				Uronic acid Rhamnose	Sulphur
		Rhamnose	Xylose	Galactose	Glucose		
crude	59	78	8	6	nd	0.76	33.8
EtOH soluble	0	2.5	0.1	1.6	0	0.00	13.8
EtOH Insoluble	143	184	16	12	4	0.78	56.6
600–400 kDa	245	345	24	11	nd	0.71	64.6
60–40 kDa	215	325	25	18	nd	0.66	55.9

nd. not determined

The Fourier Transform infrared (FTIR) analysis of all fractions, except the ethanol-soluble one, showed the typical absorption spectra of molecular linkages present in uronic acids and sulfated rhamnose. On the 600–400 kDa fraction FTIR spectra (Figure 1(b)), the peaks at 847, 1052, 1256, 1641, and 3446  $\text{cm}^{-1}$  are caused by the bending vibration of C–O–S in axial position, the stretching vibration of C–O, S=O, C=O: and C–OH, respectively. Absorbance

ratio between signals observed at 1256  $\text{cm}^{-1}$  (S=O) and 1641  $\text{cm}^{-1}$  (C=O) were higher than 0.9 for the ethanol-insoluble and derived fractions (the 600–400 kDa and the 60–40 kDa fractions) while it was only 0.26 for the ethanol-soluble fraction (Table 2). The high values compared to the one for the crude extract suggested that the obtained fractions were highly enriched in a sulfated compound, ulvan.

TABLE 2: Ratio of sulfate and carboxyl groups deduced from the FTIR spectra of ulvan-containing fractions. Signals observed at 1256 and 1641  $\text{cm}^{-1}$  are caused by the stretching vibration of the S=O of sulfated rhamnose and of the C=O of uronic acid, respectively.

Fraction	S=O
	C=O
Crude	0.68
EtOH soluble	0.26
EtOH insoluble	0.93
600–400 kDa	0.97
60–40 kDa	0.96

To look for a potential elicitor activity, the various fractions were infiltrated into the leaves of transgenic tobacco plants expressing the *uidA* reporter gene (encoding the enzyme  $\beta$ -glucuronidase, *GUS*) fused to the 2.3 kb regulatory promoter region of the tobacco lipoxygenase gene *NtLOX1* (Fanmartino et al. [24]). *NtLOX1* is induced in response to elicitor and jasmonic acid treatment as well as during infection with an oomycete pathogen [25]. Infiltration of ethanol-insoluble, 600–400 kDa and 60–40 kDa fractions ( $1 \text{ mg} \cdot \text{ml}^{-1}$ ) induced a strong *GUS* activity whereas no induction was observed with the ethanol-soluble fraction (Figure 1(c)). These results showed that the lipoxygenase-inducing activity is positively correlated with the presence of ulvan in the fractions.

**3.2. Ulvan Preparation Induces Transcriptome Changes Similar to Those Induced by Methyl Jasmonate in the Model Legume *Medicago truncatula*.** The legume *M. truncatula* was previously shown to be a useful model to study the elicitor activity of *Ulva* extract [14]. Thus we used this model plant to investigate transcriptomic changes in response to ulvan extracts (ethanol-insoluble fraction,  $1 \text{ mg} \cdot \text{ml}^{-1}$ ) and compare them to the variations observed upon treatment with two well-known mediators of plant defense, the salicylic acid analog acibenzolar-S-methyl (BTH,  $40 \mu\text{g} \cdot \text{ml}^{-1}$ ) and methyl jasmonate (MeJA,  $500 \mu\text{M}$ ). Four-week old *M. truncatula* plants were treated by pulverisation on the aerial part. Total RNA samples prepared from the leaves 1 and 2 days after treatment and pooled. Other samples were collected 6 days after treatment. Two independent biological replicates were performed and two extractions of total RNA were performed for each pool of plants. Microarrays were performed on Mt16kOLI1Plus chips representing about 16,000 open reading frames of *M. truncatula* [26]. After ANOVA analysis, a gene was considered responsive to the treatments if it associated with a *P*-value  $\leq .05$  and regarded as differentially expressed if the absolute value of  $\log_2$  ratio was  $\geq 0.584$  ( $> 1.5$ -fold induction or  $< 0.67$  repression).

Ulvan treatment induced 317 genes and repressed 146 genes among which the most induced ( $> 2$ -fold induction) and most repressed genes ( $< 0.5$ -fold) are listed in Tables S1 and S2 in supplementary material available on line at doi: 10.1155/2010/525291. More than 40% of the differentially expressed genes were found to be affected by MeJA whereas

only 10% were common with BTH treatment (Figure 2(a)). The genes coexpressed by Ulvan and MeJA are related to various functional classes (Supplementary Tables S3 and S4), notably some well-known jasmonic acid-responsive genes (Table 3) belonging to defense responses, including lipoxygenase, hydroxyproline rich glycoproteins, proline rich proteins, cysteine rich antifungal proteins (defensin) and wound induced protein [27]. Other genes also regulated by this pathway such as pectinesterase inhibitors, peroxidase, and GDSL lipase [28–30] were induced by ulvan.

Analysis of the microarray results suggested that ulvan treatment could also affect other hormonal pathways which have been shown to interfere with defense reaction [5]. A GH3-auxin responsive gene (MT000633) was found highly induced upon ulvan treatment as well as a protein phosphatase type 2C (PP2C; MT011314) (Supplementary Table S1). It has been shown that PP2Cs acts in *Arabidopsis* as negative regulator of the ABA response. Two genes coding GRAS transcription factors were also induced by ulvan. GRAS factors are involved in various processes in plants including the response to gibberellins [31]. Interestingly, this family of transcriptional factors has been also involved in response to bacterial and fungal elicitors, providing evidence for a cross-talk between elicitor and gibberellin response [32, 33].

Among typical plant responses to JA is the induction of trypsin inhibitory activity [34]. Therefore, we measured the antiprotease activity of *M. truncatula* leaves 6 days after plant treatment with ulvan extract ( $1 \text{ mg} \cdot \text{ml}^{-1}$ ), MeJA ( $500 \mu\text{M}$ ), and BTH ( $40 \mu\text{g} \cdot \text{ml}^{-1}$ ). The trypsin inhibitory activity was found to be induced upon ulvan and MeJA whereas no effect of BTH treatment was observed (Figure 2(b)). This strongly reinforced the notion that ulvan acts through a jasmonic acid signaling pathway in *M. truncatula*.

Since the transcriptomic data indicated an effect of ulvan on the hormonal balance, the level of SA, JA, and ABA was measured by a method allowing the simultaneous detection of these compounds in a single extract (see Section 2). Whereas a slight decline of free SA contents was observed, ulvan induced a transient elevation of JA content (Figure 2(c)). In addition, a transient decrease of free ABA concentration was also observed.

**3.3. The Elicitor Activity of Ulvan Depends on *JAR1* in *A. thaliana*.** The effect of ulvan on signaling and defense was additionally studied in *A. thaliana* by following the expression of *PDF1.2*, a JA responsive defensin gene, as well as *PR1a* and *PR5*, two SA responsive genes. Ulvan ( $1 \text{ mg} \cdot \text{ml}^{-1}$ ) was sprayed onto the leaves of four-week old-plants and gene expression was measured 5 days later. As shown in Figure 3(a), *PDF1.2* was highly induced in response to ulvan, an effect which was strongly reduced in the *jasmonic acid resistant*, *jar1.1* and the *abscisic acid deficient*, *aba3.1* mutants. In contrast, expression of the *PR1a* and *PR5* genes, two SA responsive genes, was not induced by ulvan and was slightly repressed in the case of the *PR1a* gene. The same kind of experiment was done by using the *salicylic acid-deficient* *NahG* line. A more intense induction of *PDF1.2* upon ulvan

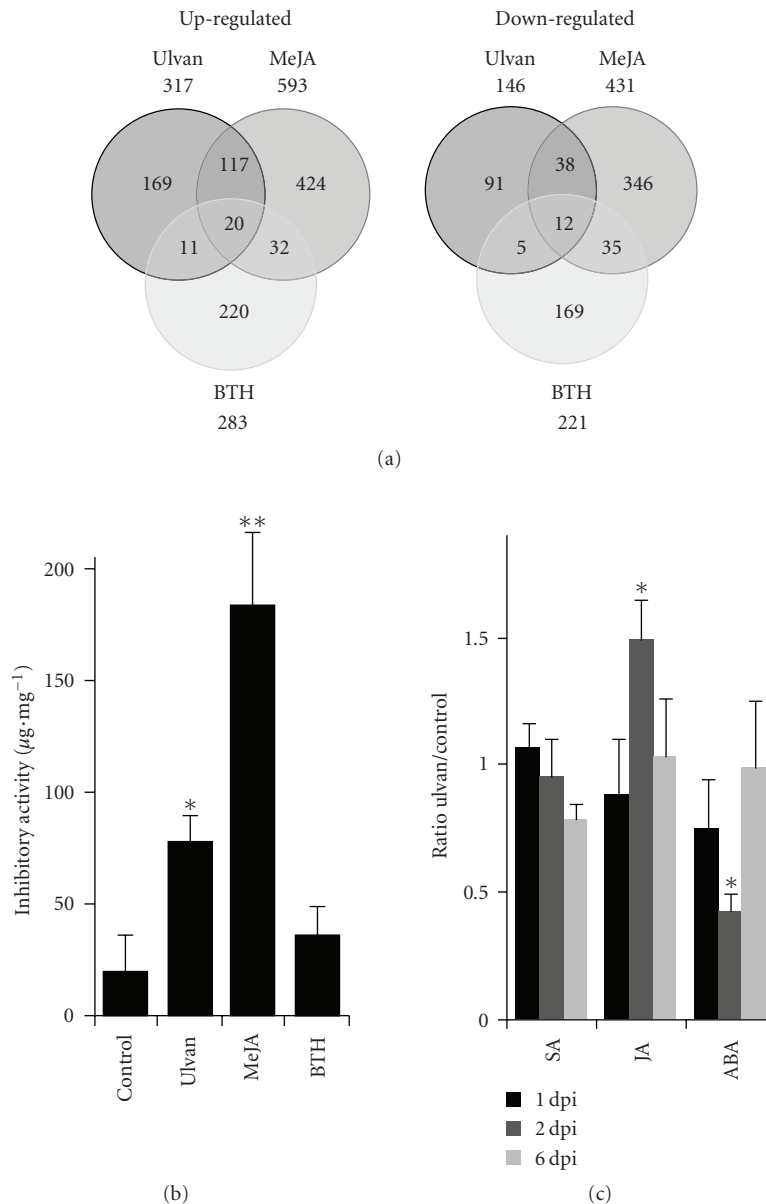


FIGURE 2: Comparison of *M. truncatula* transcriptomic responses to ulvan, BTH, and MeJA. *M. truncatula* plants were treated by ulvan (ethanol-insoluble fraction,  $1\text{ mg}\cdot\text{ml}^{-1}$ ), MeJA ( $500\ \mu\text{M}$ ), BTH ( $40\ \mu\text{g}\cdot\text{ml}^{-1}$ ), or Actilandes ( $0.01\%$ ) as the control. (a) Venn diagrams showing the total number of genes up-regulated (left) or down-regulated (right) upon ulvan, BTH, and MeJA treatments and their respective overlaps. Data obtained at 1–2 and 6 days after treatment are mixed. Only genes with a  $P$ -value  $\leq .05$  and a  $|\log(2)$  ratio  $\geq 0.584$  were selected. (b) Trypsin protease inhibiting activity measured on *M. truncatula* leaves harvested 6 days after treatment. Activity is expressed as  $\mu\text{g}$  of trypsin inhibited per mg of protein extracted from the leaves. The average of measures done on three biological replicates is represented. (c) Measurement of the SA, JA, and ABA contents of *M. truncatula* leaves harvested 1, 2, and 6 days after treatment using UPLC-ESI-MS/MS. Mean of the relative amount after ulvan treatment compared to Actilandes, used as control, was calculated from three experiments and represented on the graph. Mean for each treatment was compared to the control mean using a Student's  $t$ -test. Treatment associated to a  $P$ -value  $\leq .05$  or  $\leq .01$  was marked, respectively, by \* or \*\*.

treatment was found in the *NahG* line than in the wild-type. Accordingly, expression of the *PR1a* and *PR5* genes was induced in the *NahG* line suggesting that these genes can be regulated by an SA-independent mechanism.

To confirm the absence of *PR1a* gene induction, leaves of a *PR1a::GUS* transgenic line [17] were infiltrated with ulvan or a *Phytophthora parasitica* elicitor (P2), known to induce

the SA signaling pathway [10]. As shown in Figure 3(b), a strong induction was observed in the case of P2 infiltration, whereas a GUS activity similar to the control was obtained upon ulvan infiltration. We conclude from these experiments that ulvan affects defense in *A. thaliana* through the JA signaling pathway, which acts synergistically with the ABA pathway and is antagonized by the SA signaling pathways.

TABLE 3: Defense genes upregulated after ulvan or MeJA treatments of *M. truncatula*.

ID-Mt16kPlus	TIGR Gene Index v8	Annotation	Ratio		P-value	
			Ulvan	MeJA	Ulvan	MeJA
MT004351	TC104560	Phospholipase D	1.72	1.66	0.036	0.046
MT014135	BF640256	Lipoxygenase	1.51	1.50	0.011	0.011
MT015543	TC107737	Wound-induced protein	1.54	1.57	0.038	0.031
MT006789	TC104515	Defensin	2.31	1.85	0.001	0.006
MT008333	TC95553	HRGP-like	2.08	2.01	0.032	0.039
MT007566	TC100849	Pectinesterase inhibitor	1.80	1.51	0.003	0.022
MT000246	TC94445	Trypsin protease inhibitor	37.31	6.41	0.010	0.018
MT000087	TC106371	Protease inhibitor	1.83	1.73	0.026	0.020
MT005382	TC111698	Phytochelatin synthetase-like protein	1.55	1.75	0.015	0.003
MT014728	TC131819	Peroxidase	1.68	1.67	0.030	0.025
MT002340	TC96537	Thioredoxin reductase	1.75	1.74	0.035	0.038
MT004461	TC95580	Heat shock protein	1.67	2.16	0.015	0.001
MT015962	TC105992	Lipase GDSL	1.51	1.52	0.037	0.034
MT000987	TC101355	Anthranilate N-benzoyltransferase-like	1.51	1.52	0.013	0.011
MT014991	TC99521	Tropinone reductase	1.53	1.77	0.014	0.002
MT006133	TC98074	4-coumarate-CoA ligase	1.71	1.85	0.001	0.000

*M. truncatula* plants were sprayed with ulvan (ethanol-insoluble fraction at 1 mg·mL<sup>-1</sup>) or MeJA (500 μM) with Actilandes at 0.1%. Leaves collected 1 and 2 days after treatment were pooled and other samples were collected after 6 days. The fold ratio is calculated with the corresponding Actilandes-treated sample.

#### 4. Discussion

In a previous work, we reported on the elicitor effect of a crude *Ulva* spp. extract on plant defense [14]. The present study was undertaken in order to identify (i) the active molecules of the extract, (ii) the transcriptomic signature induced by ulvan treatment, and (iii) the plant signaling pathways involved in the response of the plants to ulvan.

From an algal crude extract, active molecules were fractionated according to their size, solubility, and activity. Active fractions were selected on the basis of their ability to induce, in transgenic tobacco, a reporter *GUS* gene construction under the control of the elicitor-inducible *NtLOX1* gene promoter (Fanmartino et al. [24]). The major recovered active compound was identified as ulvan, a polysaccharide specifically found in green algae. Its molecular mass distribution, sugar composition, and infrared spectra were similar to previous data reported on purified ulvan. These include the molecular mass distribution into high (1200–300 kDa) and medium (180–85 kDa) molecular mass populations [35], the amounts of uronic acid and rhamnose, 36 mol% and 55 mol%, respectively [36–38] and the typical absorption spectra of molecular linkages present on ulvan [39]. Ulvan is a complex branched polysaccharide composed of uronic acids (galacturonic acid, iduronic acid) and neutral sugars (rhamnose, galactose, xylose) which can be sulfated (rhamnose). Ulvan extracted from natural source may also contain divalent cations such as Ca<sup>2+</sup>. The high molecular weight fraction we used in our experiments contained mainly a polysaccharide with a monosaccharide composition typical of ulvan (rhamnose, uronic acid, and xylose, galactose) and sulfate. These components account for 65% of the fraction dry mass. However, this probably

indicates a partial hydrolysis of the polymer since it has been shown that ulvan is partially resistant to TFA hydrolysis [38]. Finally, the FTIR spectra done on the purified fraction indicates linkages typically found in ulvan. All these data strongly suggest that the fraction is, at least, highly enriched in ulvan and this preparation does not contain contaminants such as proteins or secondary metabolites. Interestingly, the mean molecular size of ulvan was reduced to about 50 kDa, corresponding to ulvan fragments of about 100 residues without modification of the biological activity.

The transcriptome of *Medicago* was largely affected by ulvan treatment and this analysis showed a strong similarity between MeJA and Ulvan responses. Accordingly, an increase of proteinase inhibitory activity, a typical MeJA responsive marker, was observed upon ulvan treatment. In addition, ulvan induces expression of JA-dependent genes like the *PDF1.2* defensin in *A. thaliana* and the lipoxygenase *NtLOX1* promoter in *N. tabacum* [40, 41]. Importantly, induction of *PDF1.2* in *A. thaliana* was dependent on *JAR1*, an essential gene in the JA signaling pathway. No induction of typical SA-dependent defense responses was observed upon ulvan treatment, such as SA accumulation in *M. truncatula* tissues or PR1a and PR5 expression in *A. thaliana*. Together, these results show that ulvan elicits a JA signaling pathway but not an SA-dependent pathway in plants belonging to three botanic families, *Nicotianae tabacum* (Solanaceae), *Arabidopsis thaliana* (Brassicaceae) and *Medicago truncatula* (Fabaceae). Fucan and carrageenans, two sulfated polysaccharides extracted from algae, were previously shown to induce lipoxygenase gene expression in tobacco [9, 10] suggesting that activation of JA pathway could be a general feature of sulfated oligosaccharides. The measure of SA, JA



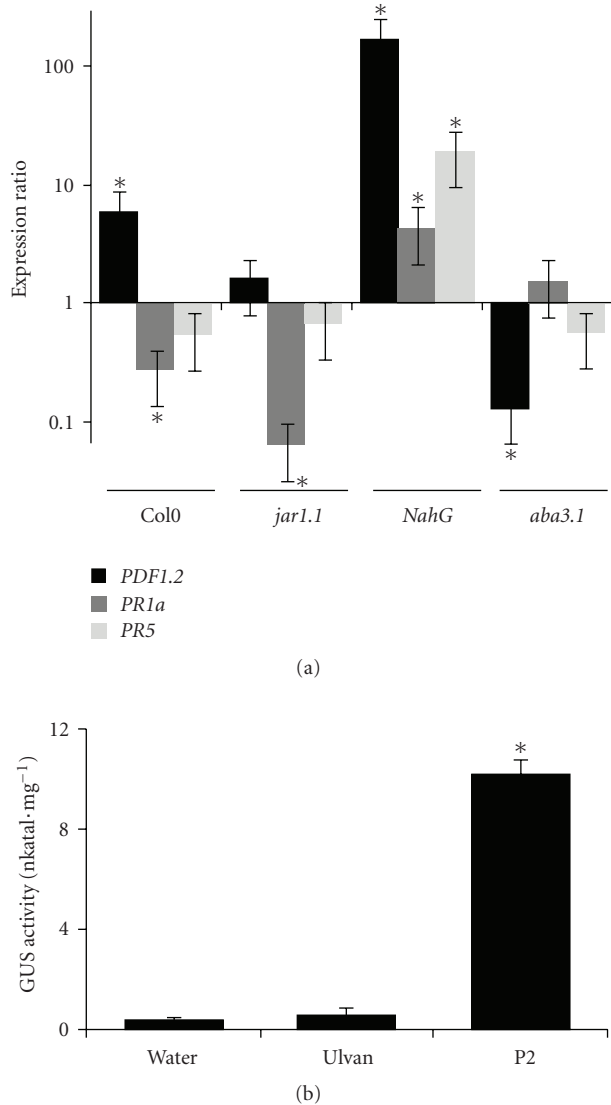


FIGURE 3: Expression of *A. thaliana* defense genes in response to ulvan. (a) RT-PCR analysis of *PDF1.2*, *PR1a* and *PR5* expression in Col0, *jar1.1*, *NahG* and *aba3.1* *A. thaliana* 5 days after ulvan treatment (ethanol-insoluble fraction, 1 mg·ml<sup>-1</sup>). Data was normalized using  $\beta$ -*Tubulin* expression. Fold ratio is calculated with the corresponding control sample. Three biological replicates showed consistent results. (b) Fluorometric measures of *GUS* activities in leaves of transgenic *Arabidopsis* expressing a *PR1::GUS* fusion. Plants were infiltrated by water, ulvan (ethanol-insoluble fraction, 1 mg·ml<sup>-1</sup>), or P2 (*Phytophthora parasitica* cell wall extract, 500  $\mu$ g·ml<sup>-1</sup>). *GUS* activity was determined 1 day post infiltration and quantified using fluorescent 4-methylumbelliferone as substrate. Mean and standard errors were calculated from three independent replicates. Specific activity is expressed as nkatal per mg of total proteins. Mean for each treatment was compared to the control mean using a Student's *t*-test. Treatment associated to a *P*-value  $\leq .05$  or  $\leq .01$  was marked, respectively, by \* or \*\*.

and ABA concentration upon ulvan treatment showed that SA concentration was unaffected whereas an increase of JA concentration and a decrease of ABA concentration were observed. Since an antagonist effect between the JA and

ABA pathway has been suggested [42, 43], the decrease of free ABA concentration should result in an increase of the JA-dependent response. In contrast, *PDF1.2* induction in response to ulvan was impaired on the ABA-mutant suggesting that ABA was also required to JA-dependent signaling pathway activation. It was recently found that *Arabidopsis* lines mutated in ABA synthesis genes are impaired in JA accumulation upon bacterial infection, suggesting that ABA can promote JA signaling pathway [44].

Ulvan is a unique polysaccharide found exclusively in green algae, raising the question of its recognition and perception by plant cell. In human and murine cells, ulvan induces the expression of several chemokine and interleukin genes [45]. Such a biological activity has been correlated to structural homologies between ulvan and glycosaminoglycans (GAGs) as they both contain sulfated sugars. GAGs, such as chondroitin sulfate have been reported to be inflammatory mediators activating monocytes [46]. In plants, ulvan might mimic the structure of elicitors containing rhamnose and/or uronic acid residues. Rhamnose is present in plant polysaccharides, such as the rhamnogalacturonan I domain of pectic polysaccharide [47] and in phytopathogenic bacterial rhamnolipids initially identified in *Pseudomonas aeruginosa*. These two rhamnose-containing compounds trigger defense responses [48, 49]. However, molecular mechanisms underlying their perception remain to be determined.

In conclusion, this study provides a thorough analysis of the elicitor activity of an *Ulva* spp. extract. We identified the active molecules of the extract as ulvan, a typical polysaccharide of green algae with potential biotechnological applications. Ulvan could be efficient against necrotrophic pathogens because it activates JA signaling pathway which has been shown to be involved against this type of microorganisms [50]. We have previously reported the efficiency of a crude *Ulva* extract against the hemibiotrophic fungus *Colletotrichum trifolii* infecting *M. truncatula* [14]. Accordingly, *Ulva* extract has also been found to protect *Malus domestica* from *C. gloeosporioides* infection [51]. More importantly, greenhouse trials, recently, conducted with artificial inoculation of melon crops with the powdery mildew pathogen *Erysiphe cichoracearum*, revealed a clear protection conferred by ulvan (Jaulneau et al., unpublished observations). Further work will aim at dissecting more precisely the molecular mechanisms underlying the perception of ulvan in order to optimize its potential use as plant protectant.

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